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Zebrafish *sox9b* is crucial for hepatopancreatic duct development and pancreatic endocrine cell regeneration

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ABSTRACT

Recent zebrafish studies have shown that the late appearing pancreatic endocrine cells are derived from pancreatic ducts but the regulatory factors involved are still largely unknown. Here, we show that the zebrafish *sox9b* gene is expressed in pancreatic ducts where it labels the pancreatic Notch-responsive cells previously shown to be progenitors. Inactivation of *sox9b* disturbs duct formation and impairs regeneration of beta cells from these ducts in larvae. *sox9b* expression in the midtrunk endoderm appears at the junction of the hepatic and ventral pancreatic buds and, by the end of embryogenesis, labels the hepatopancreatic ductal system as well as the intrapancreatic and intrahepatic ducts. Ductal morphogenesis and differentiation are specifically disrupted in *sox9b* mutants, with the dysmorphic hepatopancreatic ducts containing misdifferentiated hepatocyte-like and pancreatic-like cells. We also show that maintenance of *sox9b* expression in the extrapancreatic and intrapancreatic ducts requires FGF and Notch activity, respectively, both pathways known to prevent excessive endocrine differentiation in these ducts. Furthermore, beta cell recovery after specific ablation is severely compromised in *sox9b* mutant larvae. Our data position *sox9b* as a key player in the generation of secondary endocrine cells deriving from pancreatic ducts in zebrafish.

Introduction

Stimulating *in vivo* regeneration of insulin-producing cells (beta cells) from endogenous precursors offers great interest in the treatment of diabetes mellitus. It has long been thought that endogenous beta cell precursors reside in adult pancreatic ducts (Bonner-Weir et al., 2004). However, this hypothesis has been recently challenged by lineage tracing studies in mouse using two ductal markers, Hnf1β (Solar et al., 2009) and Sox9 (Kopp et al., 2011; Solar et al., 2009), which have failed to highlight endocrine differentiation from pancreatic ducts after birth. In contrast, in zebrafish, there is accumulating evidence of ductal origin of endocrine cells after embryogenesis (Dong et al., 2007, 2008; Field et al., 2003; Parsons et al., 2009; Wang et al., 2011). Thus, it is crucial to understand how beta cells are generated from ducts in zebrafish if we hope to stimulate this pathway in mammals to be able to provide diabetes patients with endogenous sources of beta cells.

Zebrafish and mammalian pancreas consist of endocrine islets embedded in a large exocrine tissue. Their development share many key steps and players (Kinkel and Prince, 2009 for review). For example, they form as two embryonic buds from the *pdx1*-expressing domain of the endoderm adjacent to the hepatic primordium. During zebrafish development, the pdx1 domain gives rise to the dorsal pancreatic bud which generates the first endocrine cells from 15 hpf, and to the ventral pancreatic anlage from 32 hpf (Biemar et al., 2001; Field et al., 2003; Roy et al., 2001). At the end of embryogenesis (3 days post fertilisation; 3 dpf), the zebrafish pancreas consists of a principal endocrine islet mostly derived from the dorsal bud surrounded by the exocrine tissue derived from the ventral pancreas and composed of acini and ducts. Along the extrapancreatic duct (EPD), between 2 and 5 dpf, additional endocrine cells appear that will join the principal islet (Dong et al., 2007, 2008; Field et al., 2003;

Abbreviations: BMP, bone morphogenic protein; EHD, extrahepatic duct; EPD, extrapancreatic duct; FGF, fibroblasts growth factor; GB, gall bladder; HNF, hepatocyte nuclear factor; HPD, hepatopancreatic ductal system; IHD, intrahepatic ducts; IPD, intrapancreatic ducts; SOX9, SRY-related HMG box transcription factor 9

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Pisharath et al., 2007) and, along the intrapancreatic ducts (IPD), secondary endocrine islets will differentiate after 5 dpf (Parsons et al., 2009; Wang et al., 2011) from a population of pancreatic progenitors, the Notch-responsive cells (PNCs), aligned along the IPD. Collectively, these late endocrine cells, derived from the ventral pancreatic bud, constitute the second wave of endocrine cell differentiation. This will ultimately contribute to the majority of the endocrine mass as the dorsal bud-derived islet cells are quiescent (Hesselson et al., 2009). Zebrafish also display a remarkable capacity to regenerate beta cells after targeted cell ablation in young larvae and adults (Curado et al., 2007; Moss et al., 2009; Pisharath et al., 2007). Whether this regeneration occurs from pancreatic ducts remains unknown.

Similar to the organisation in amniotes, the pancreatic ducts in zebrafish form a complex branched network which is anatomically connected to the intestine and the hepatic ducts through the hepatopancreatic ductal system (HPD) (Dong et al., 2007). In mammals, SOX9 is expressed in all these ductal structures from embryogenesis to adulthood (Antoniou et al., 2009; Carpentier et al., 2011; Furuyama et al., 2011; Kopp et al., 2011; Seymour et al., 2007) but its function in their ontogenesis is still poorly explored. In the mouse liver, SOX9 is the earliest marker of intrahepatic ductal cells (also called cholangiocytes) and is involved in the maturation of these ducts (Antoniou et al., 2009). In the mouse pancreas, SOX9 is detected in the progenitor cells of the pancreatic anlagen (Lynn et al., 2007; Seymour et al., 2007) and its inactivation causes pancreas hypoplasia with severe reduction of all pancreatic cell types, endocrine, acinar and ductal (Seymour et al., 2007). However, its function specifically in pancreatic ductal development remains unknown, as well as its role in the formation of the HPD. In the present study, we show that zebrafish *sox9b* gene expression defines a novel population of progenitors partially overlapping the adjacent hepatic and pancreatic anlagen and is later expressed in the intrahepatic, intrapancreatic ducts and in the HPD system. Using a new *sox9b* mutant, the *sox9b*^{fh313}, we demonstrate that *sox9b* plays an essential role not only in intrahepatic duct morphogenesis but also in morphogenesis and differentiation of the intrapancreatic ducts and of the HPD system. Finally, we show that *sox9b* is essential for endocrine cell formation from the pancreatic ducts and for beta cell regeneration.

Material and methods

Zebrafish strains

Embryos and adult fish were raised and maintained under standard conditions. We used the following transgenic and mutant lines: *sox9b*^{fh313} identified by TILLING (Draper et al., 2004) (http://www.zfishtilling.org/zfish/index.php), *sox9b*^{b971} (Yan et al., 2005), *Tg*(*ptf1a*:*egfp*)^{jhl} (Godinho et al., 2005), *daedalus* (*dae*^{tbvbo} mutant allele of *fgf10*) (Norton et al., 2005), *Tg*(*Tp1*:*hmgb1-mCherry*) (Parsons et al., 2009) and *Tg*(*ins*:*nfsB-mCherry*) (Pisharath et al., 2007).

Adult *sox9b*^{fh313} heterozygous and mutant embryos in their progeny were identified by genotyping.

Sorting of GFP+cells by FACS

Fluorescent cells were isolated from pancreas dissected from adult *Tg*(*Tp1*:*hmgb1-eGFP*) and *Tg*(*ptf1a*:*eGFP*) zebrafish. Flow cytometry was performed using a FACSAria (Becton Dickinson) flow cytometer.

EdU injection and labelling

The Click-iT EdU Alexa Fluor555 Imaging Kit (Invitrogen) was performed following the manufacturer's protocol. Briefly, 5 nl of EdU solution (1 μM EdU, 2% DMSO, 0.1% phenol red) were injected into the yolk of 3 or 4 dpf larvae which were further incubated 60 min and fixed in 2% PFA. Larvae were then processed for 2F11-duct immunodetection and nuclei staining with DRAQ7 (Biostatus Limited).

Whole mount in situ hybridisation and immunohistochemistry

Fluorescent and colorimetric whole mount *in situ* hybridisation was performed as previously described (Hauptmann and Gerster, 1994; Manfroid et al., 2007) with the following probes: *sox9b* (Yan et al., 2005), *neurod* (Korzh et al., 1998), *pdx1* (Milewski et al., 1998), *cp* (Korzh et al., 2001), *try* (Biemar et al., 2001), *ptf1a* (Zecchin et al., 2004), *tfa* (Mudumana et al., 2004) and *prox1a* (Glasgow and Tomarev, 1998).

Whole mount immunohistochemistry was described in Dong et al. (2007). We used the following antibodies: polyclonal rabbit anti-Prox1 (1:1000, Chemicon), polyclonal guinea pig against zebrafish Pdx1 (1:200, gift from C. Wright), polyclonal goat anti-HNF4α (1:100, Santa Cruz Biotechnology), monoclonal mouse 2F11 (1:1000, Abcam ab71286) (Crosnier et al., 2005), rabbit polyclonal anti-Pax6a/b (1:500, kind gift of F. Biemar and D. Georlette), mouse monoclonal anti-Nkx6.1 (1:15, developed by O.D. Madsen and obtained from the Developmental Studies Hybridoma Bank, F55A1), chicken anti-GFP (1:1000, Aves Labs), rabbit polyclonal anti-SOX9 (1:500, gift from Silvana Guioli) (Morais da Silva et al., 1996), mouse monoclonal anti-mCherry (1:400, anti-DsRed from Clontech) and fluorescently conjugated AlexaFluor antibodies (Invitrogen).

Fluorescent images were acquired with a Leica SP2 or Olympus FluoView FV1000 confocal microscopes.

Genotyping of sox9b, fgf10 and fgf24 mutants

Genotyping was performed on genomic DNA extracted from adult tails or tails obtained from embryos processed through *in situ* hybridisation or immunohistochemistry. The *ikarus* mutation in the *fgf24* locus generates a restriction site for the *AccI* endonuclease. The PCR fragment obtained with forward 5′-CTGTCAGTCCCACAGCAGTGGACCA-3′ and reverse 5′-CCATGTA-GATTTTATTACATGTAGGT-3′ primers (615 pb) digested by *AccI* produces two fragments (185 and 430 pb) in the mutants. The *daedalus* mutation in *fgf10* generates a SNP that was identified by the TaqMan SNP Genotyping Assays (Applied Biosystem). The region encompassing the mutation was amplified with the forward primer dae-SNp1F 5′-CCGAGCTCCAGGACAATGTG-3′ and reverse primer dae-SNp1R 5′-GCAGGACAGACGGAACCA-3′. Allelic discrimination was performed by dae-SNp1V2 VIC primer 5′-CCCTTAGTCACTTTCCATTT-3′ (wild type allele) and dae-SNp1M2 FAM primer 5′-CCTTAGTCACTTaCCATTT-3′ (mutant allele) according to the manufacturer.

The *sox9b*^{fh313} mutation creates a *SfcI* restriction site. A 440 pb PCR fragment containing the mutated site was amplified with forward 5′-TGTCCGGAGCTCCGAGCCCGAG-3′ and reverse 5′-ACT-CATCAGTGCCCTTACTMAGTGTG-3′ primers. Upon digestion, the presence of the mutation generated two fragments (166 and 274 pb).

Treatments with pharmacological inhibitors and beta cell ablation and recovery

Wild type embryos were treated with 5 μM SU5402 (Calbiochem) from 54 to 72 hpf or with 100 μM γ-secretase inhibitor DAPT from 3 to 4 or 6 dpf (Calbiochem). DMSO was used in control treatments.

For specific beta cell ablation, *Tg*(*ins*:*nfsB-mCherry*); *sox9b* compound larvae were treated at 56 hpf with 7.5 mM Metronidazole

(Sigma) with 0.1% DMSO for 24 h as previously described (Pisharath et al., 2007). This concentration does not cause any adverse effect in the zebrafish larvae. After several washes with fish water, some larvae were fixed in 1% PFA to check mCherry fluorescence and ablation efficiency. The other larvae were grown up to 6.5 dpf, fixed in 2% PFA and processed for 2F11 immunohsitochemistry (revealed with anti-mouse AlexaFluor488, Invitrogen). As we could not easily detect mCherry fluorescence after IHC process, insulin immunodetection was performed along with 2F11, and was revealed by anti-guinea pig AlexaFluor555 (Invitrogen). Larvae were then genotyped for identification of *sox9b* mutants and examined with confocal microscopy.

Results

Zebrafish sox9b is first expressed in the hepatopancreatic endoderm and then in the entire hepatopancreatic ductal system

While expression of the zebrafish *sox9a* and *sox9b* genes has been reported at the level of the neural crest, pharyngeal arches, otic vesicles, somites, central nervous system and gonads (Akiyama et al., 2005; Chiang et al., 2001; Cresko et al., 2003; Piper et al., 2002; Yan et al., 2005), no data was available so far on their expression in the liver and in pancreas. Hence, we analysed in detail the endodermal expression of the two zebrafish *Sox9* paralog genes by *in situ* hybridisation. *sox9b* transcripts were detected in the hepatopancreatic region in contrast to *sox9a* which was never detected in the midtrunk endoderm at all examined stages. From 24 hpf, *sox9b* is expressed at the base of the dorsal pancreas and, just anteriorly, in the region of the hepatic primordium and of the prospective ventral pancreas (abbreviated HVP for Hepatic-Ventral Pancreatic primordia) (Fig. 1A, D, E). While expression at the level of the dorsal pancreatic bud is transient, over the next day of development, *sox9b*+ cells outline a duct-like structure linking the liver and pancreas from 2 dpf (Fig. 1B, C). To define more precisely the structures expressing *sox9b*, double fluorescent labelling was performed between 28 and 80 hpf with hepatic and pancreatic markers. At 28 hpf, *sox9b* expression in the HVP region overlaps the caudal part of the *prox1a*+ hepatic domain (Fig. 1D) and the anterior part of the *pdx1*+ pancreatic domain (Fig. 1E), indicating that *sox9b* is expressed at the boundary of the two domains. At that stage, some *prox1a*+/*pdx1*+ cells were detected at this

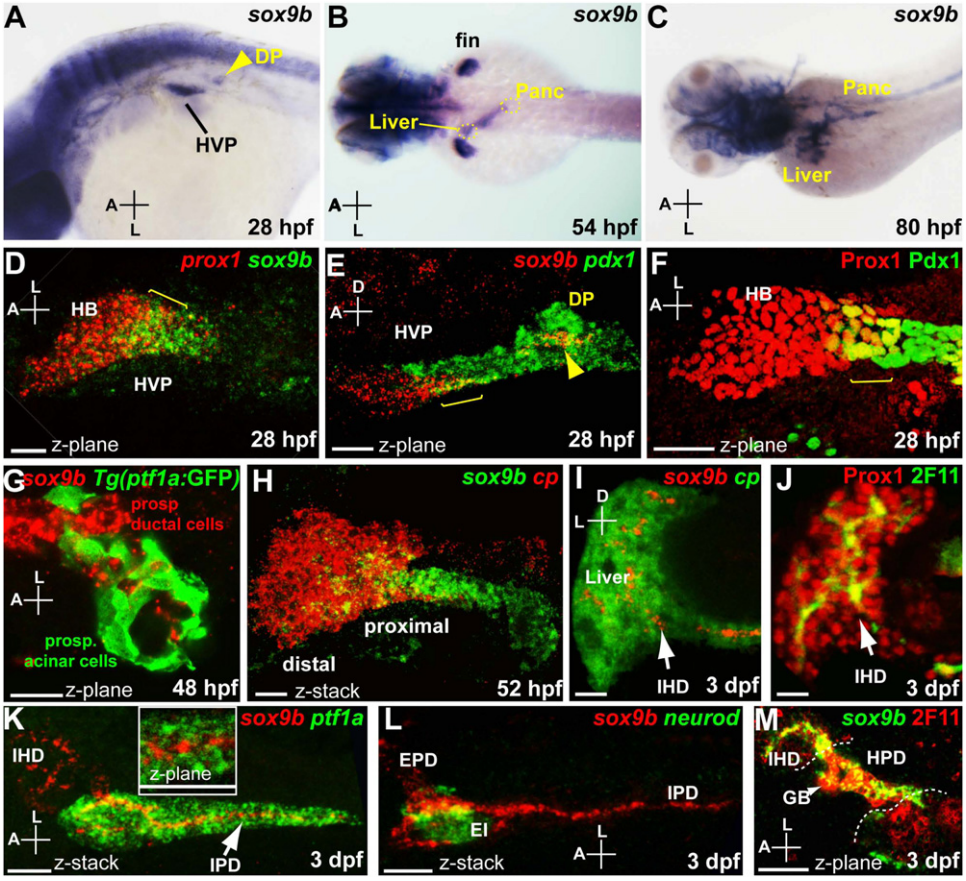

Fig. 1. *sox9b* expression in the HPD system, IPD and IHD throughout zebrafish embryonic development. (A) *sox9b* transcript is detected in the region of the hepatic and ventral pancreatic primordia (HVP). The yellow arrowhead points to the dorsal pancreas (DP). (B,C) During embryogenesis, *sox9b* expression delineates a network connecting the liver and pancreas. Yellow dotted lines delimit the pancreas and the liver primordia. (D) Confocal section through *sox9b* and *prox1a* expression in the HVP domain at 28 hpf. The brackets highlight the overlap between both expression domains. (E) Confocal section through *sox9b* and *pdx1* adjacent and partially common expression domains. (F) Immunodetection of Prox1 and Pdx1 revealing a partial overlap (bracket). (G) *sox9b* expression in the ventral pancreas of *Tg*(*ptf1a*:*eGFP*) embryos. (H) Confocal projection of *sox9b* and the hepatocyte marker *cp* expression in the hepatic bud at 52 hpf. (I, J) Comparison at 3 dpf of *sox9b* expression in the liver labelled by *cp* (I) with the pattern of the IHD labelled with the ductal marker 2F11 and Prox1 (the IHDs appear yellow) (J). *sox9b* expression is restricted to *cp*-cells (I, arrows) and displays a highly similar pattern to 2F11+ cholangiocytes (J). (K) At this stage, in the pancreatic acinar tissue labelled with *ptf1a*, *sox9b* is restricted to IPD (inset) and EPD cells. (L) Detection of SOX9 protein in the Notch-responsive cells (PNCs) along the IPD of *Tg*(*Tp1:hmgb1-mCherry*) transgenic larvae at 5 dpf. (M) *sox9b* mRNA detection followed by immunolabelling with the ductal maker 2F11 demonstrating *sox9b* expression in the IHD and in the HPD system, notably in the gall bladder (GB, arrowhead). The dotted lines delineate the border of the liver and pancreas with the HPD. Note that the IPD could not be detected by 2F11 immunolabelling following *in situ* hybridisation. EHD, extrahepatic duct; EI, endocrine islet; EPD, extrapancreatic duct; GB, gall bladder; HB, hepatic bud; HVP, hepatic and ventral pancreatic domain; IHD, intrahepatic ducts; IPD, intrapancreatic ducts; OV, otic vesicle; VP: ventral pancreas. Scale bar=40 μm except H, I and *J*=20 μm.

boundary (Fig. 1F). Double staining at 2 dpf with the ventral pancreatic marker *ptf1a* (Fig. 1G) and the hepatic marker *ceruloplasmin* (*cp*) (Fig. 1H) revealed that the *sox9b*+ cells form the prospective HPD system located in the proximal part of the ventral pancreatic and hepatic buds and making a junction between these two buds (see also Fig. 1B). Within the pancreatic and hepatic buds, *sox9b* is largely excluded from *ptf1a*+ cells (prospective acinar cells) and *cp*+ hepatocytes, and is expressed in the prospective ductal cells (Fig. 1G, H). At 3 dpf, the *sox9b* cells within the liver are distinct from the *cp* labelled hepatocytes (Fig. 1I) and create a pattern similar to the IHD revealed with the ductal 2F11 antibody (Fig. 1J) (Crosnier et al., 2005; Lorent et al., 2010). Furthermore, *sox9b* and 2F11 domains perfectly overlap in the HPD system connecting the liver and pancreas, showing that *sox9b* is expressed in the EHD, EPD and the gall bladder (GB) (Fig 1M). Within the pancreas (Fig. 1K, L), *sox9b* was also detected in the IPD that surrounds the principal islet (labelled with *neurod*; Fig.1L) and extends posteriorly in the pancreatic tail.

In conclusion, these data indicate that *sox9b* is first expressed in a subset of progenitors encompassing the border between the liver progenitor domain and the prospective ventral pancreas, and, at later stages of development, its expression delineates the biliary and pancreatic ductal network and the HPD system that connects them to the intestine.

sox9b promotes morphogenesis and differentiation of the hepatic, pancreatic and hepatopancreatic ductal system

Since the previously reported *sox9b*^{b971} mutant allele (Yan et al., 2005) contains a large deletion comprising genes adjacent to *sox9b* which could interfere with the analysis of *sox9b* function, we investigated the role of *sox9b* in liver and pancreas development by analysing the phenotype of a new *sox9b* mutant allele. This mutant, *sox9b*^{fh313}, harbours a nonsense mutation (K68*) in the first exon generating a short truncated protein lacking all the conserved functional domains and therefore should not be functional. Furthermore, SOX9b protein could not be detected by immunostaining (data not shown) indicating that no alternative start codon is used downstream the mutation. In contrast to the *sox9b*^{b971} deletion mutants (Yan et al., 2005), the general morphology of the new *sox9b*^{fh313} mutant embryos is identical to wild type. At 3 dpf, the global morphology and the size of the liver and the pancreas in the *sox9b*^{fh313} mutants are indistinguishable from the wild type organs, as revealed by the *transferrin a* (*tfa*) and *trypsin* (*try*) markers (Fig. 2A, B). In addition, the endocrine islet derived from the dorsal pancreatic bud also appeared unaffected (data not shown). Analysis of *prox1a*, *pdx1* and *ptf1a* expression at 24 and 34 hpf also indicated normal specification of the hepatic and ventral pancreatic domains (data not shown). In contrast, as depicted in Fig. 2C and D, while Prox1 immunolabelling at 3 dpf confirmed that the overall morphology of the liver and pancreas is normal in *sox9b* mutants as well as the number of hepatocytes and pancreatic acinar cells, the 2F11 staining revealed dysmorphic ducts within the liver, pancreas and in the HPD system connecting the two organs to the intestine in all larvae examined. These defects were already observed at 2.5 dpf (data not shown) and they were more obvious at 7 dpf when hepatic and pancreatic ductal systems are completed and functional (compare the liver in Fig. 2 G, G′ and H, H′, and the pancreas in Fig. 2G″ and H″). In the liver of wild type embryos and larvae, while IHD cells (2F11+) are contacting one another to create a well defined and thin IHD network (Fig. 2E′ at 3 dpf and Fig. 2G, G′ at 7 dpf), they remain clustered together in the mutants and form thicker and fewer cellular interconnections (Fig. 2F′ at 3 dpf and Fig. 2H, H′ at 7 dpf, see asterisks).

In the pancreas, the antero-posterior alignment of IPD cells and the extent of their migration driving primary pancreatic duct formation within the tail of the pancreas appeared normal (compare Fig. 2C and D). In contrast, these cells display much weaker staining of the ductal marker 2F11 at 3 dpf compared to

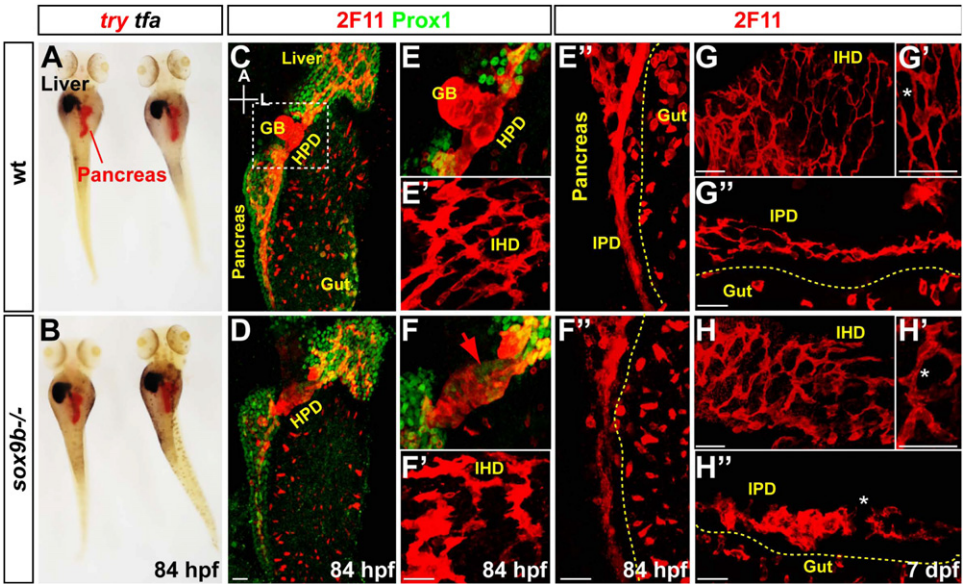

Fig. 2. Affected morphogenesis of the IPD, IHD and HPD system in *sox9b* mutants. (A, B) Acinar (*try*) and hepatocyte (*tfa*) differentation as well as the global morphology of the larvae are similar in wild type larvae (A) and in *sox9b* mutants (B) at 84 hpf. (C, D) Three dimensional rendering of the liver and pancreas at 84 hpf, labelled by Prox1, and of the entire ductal network highlighted by 2F11. The insets represent the 2F11 staining in the liver. White arrows in D and inset point at the disrupted connections between cholangiocytes in *sox9b* mutants. (E, F) Close-up of the HPD system connecting the pancreas and the liver to the intestine. Ectopic Prox1+ cells are detected throughout the HPD system (red arrow). (E′, F′) Less interconnecting ducts are detected in the IHD *sox9b* mutants. (E″, F″) 3-D rendering of the pancreas showing weaker 2F11 labelling in the IPD of *sox9b* mutant (F″) compared to wild type (E″). (G, H). 3-D rendering at 7 dpf of ductal 2F11 labelling in the liver (IHD) in wild type (G) and *sox9b* mutants (H). (G′, H′) Higher magnification showing thicker connections between IHD cells (asterisk). (G″, H″) 3-D rendering at 7 dpf of ductal 2F11 labelling in the pancreas (IPD) in wild type (G′) and *sox9b* mutants (H′). GB, gall bladder; HPD, hepatopancreatic ductal system; IHD, intrahepatic ducts; IPD, intrapancreatic ducts. Scale bar=20 μm.

wild type ducts (Fig. 2E″, F″). At 7 dpf, the primary pancreatic ductal network is discontinuous presenting masses of aggregated cells forming cyst-like structures; the ductal branching leading to the formation of first and second order pancreatic ducts are also clearly affected (Fig. 2G″, H″). In contrast, the acinar tissue is normal in the mutants (data not shown).

In addition to anomalies of the IHD and IPD, the HPD system joining the pancreas and liver to the intestine is also dysmorphic in *sox9b* mutants as it is dilated and harbours a smaller GB compared to wild type larvae (Fig. 2E, F). The HPD system also shows weaker 2F11 staining than in wild-type embryos and displays ectopic Prox1+ cells (red arrow in Fig. 2F′) at 3 dpf (see below). All these observations reveal that *sox9b* is required not only for the morphogenesis of the IHD but also for morphogenesis and differentiation of the IPD and HPD system.

To examine more closely the differentiation defect of the HPD in *sox9b* mutants, we analysed several markers at 3 dpf. As depicted in Fig. 3, while these ducts in wild type larvae are labelled by 2F11 only and not by Prox1 and HNF4α, the dysmorphic *sox9b*−/− HPD contains misdifferentiated cells at 3 dpf (see Fig. 3A, B for 3D projections of the HPD system and Fig. 3A′ and B′–B‴ for *z*-planes through the same region). Indeed, hepatic-like cells (coexpressing Prox1 and HNF4α) were found in most mutant embryos within these structures (71% of embryos, $n=14$), notably proximal to the pancreas (green arrowheads in Fig. 3B and B″). Similarly, ectopic Pax6+ pancreatic endocrine cells could be detected distal from the pancreas (Fig. 3C, D, white arrowheads), notably in the EHD and GB and even within the liver (66% of mutant embryos, $n=12$). Moreover, the pancreatic ductal marker Nkx6.1 is ectopically expressed throughout the HPD system in all mutants examined (compare Fig. 3A, A′ and Fig. 3B–B‴). Indeed, whereas in wild type larvae high level of Nkx6.1 expression was normally detected in the IPD only (see yellow cells within the pancreas in Fig. 3A, A′ as IPD cells also express Prox1 which is revealed in green), Nkx6.1+ cells were also found in all parts of the HPD in *sox9b* mutants, notably within the junction with the intestine as well as near and within the liver, *i.e.* locations far from the pancreas (yellow arrowheads in Fig. 3B, B′–B‴). Ectopic pancreatic acinar cells were never detected in the HPD system of the mutants (data not shown). Moreover, a significant number of ectopic Nkx6.1+ cells in the HPD also exhibit different levels of HNF4α, demonstrating completely aberrant differentiation of HPD duct cells resulting in chimeric identities (pink arrowheads pointing at triple Nkx6.1/Prox1/HNF4α positive cells in Fig. 3B and B‴).

As the anomalies of hepatic and pancreatic ducts could partially be due to a reduced number of duct cells, we counted cells in both organs in *sox9b* mutants at 3 and 4 dpf. The number of IHD cells and hepatocytes appeared globally unaffected in mutant livers compared to wild type. In contrast, there is a decreased number of IPD cells (94 ± 15 duct cells in $n=11$ wild type pancreas *versus* 58 ± 10 cells in $n=10$ mutants at 4 dpf) while the amount of acinar cells is unaffected, as was already suggested by normal *tryspin* expression at 3 dpf. No significant difference in proliferation (EdU and PH3 labelling, Supplemental Table 1) and apoptosis (Tunel labelling, data not shown) of duct cells could be detected in the liver and pancreas between wild type and *sox9b* mutants. Similarly, hepatocyte and acinar cell

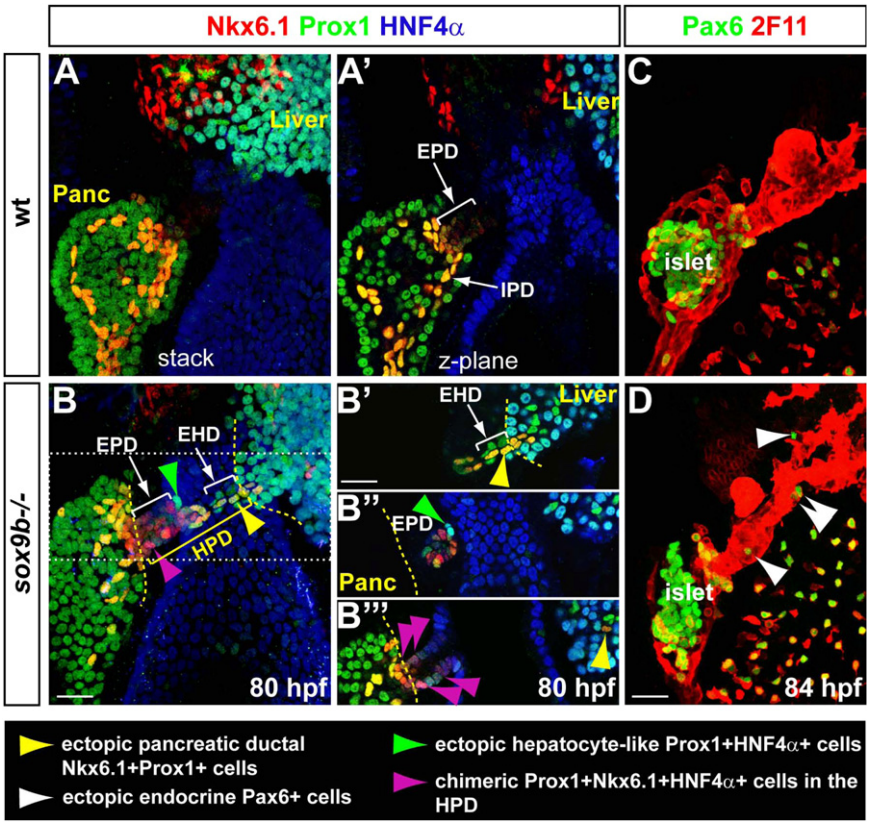

Fig. 3. Misdifferentiation of the HPD system in *sox9b* mutants. (A, B) 3D-rendering (stack) at 80 hpf of the liver, pancreas and HPD system immunolabelled with Prox1, HNF4α and the pancreatic ductal marker Nkx6.1. (A′, B′–B‴) *Z*-planes through the same larvae as in A and B. Prox1 with HNF4α label hepatocytes while Prox1 together with Nkx6.1 strongly labels the IPD. In *sox9b* mutants (B and B′–B‴), the HPD system becomes labelled with the three markers and display different ectopic cell types (see the colour code at the bottom). Dotted lines delimitate organs and ducts boundaries. (C, D) 3-D rendering of the HPD system at 84 hpf labelled with the ductal marker 2F11 and the endocrine marker Pax6. Ectopic endocrine cells along the HPD and in the liver are indicated by white arrowheads. EHD, extrahepatic duct; EPD, extrapancreatic duct; HPD, hepatopancreatic ductal system; IPD, intrapancreatic ducts. Scale bar=20 μm.

proliferation and apoptosis are not affected in the mutant. These data indicate that *sox9b* is not critical for duct cell proliferation and survival. Overall, our results suggest that it is required for establishment of proper connections between duct cells in the liver and pancreas as well as for duct cell differentiation in the pancreas and HPD system.

The *sox9b*^{fh313} mutant allele is not embryonic lethal but the *sox9b* mutants hardly reach adulthood. Indeed, the analysis of the survival rate in the progeny of *sox9b* heterozygote matings revealed only one surviving homozygous mutant out of 81 fish after 6 months, and 3 homozygotes out of 31 fish after 2.5 months. Furthermore, the overall size of the fish is dramatically reduced in the mutants (average *sox9b* mutant length 1.7 ± 0.36 cm compared with 2.42 ± 0.48 cm for wild type fish at 2.5 months).

sox9b early expression is activated by the FGF and BMP signalling pathways

In order to identify extrinsic factors required for the activation of *sox9b* expression in the hepatopancreatic domain at 24 hpf, we examined signalling pathways known to be involved in the specification of the ventral pancreas and the liver in zebrafish. Specification of the liver bud has been previously shown to depend on the action of WNT2bb (Ober et al., 2006). Also, we previously reported that the FGF signalling, namely FGF10 and FGF24, is essential for ventral pancreas specification (Manfroid et al., 2007) while BMP2a is important for both liver and ventral pancreas specification (Naye et al., 2012). All these extrinsic factors are secreted from the lateral plate mesoderm adjacent to the prospective endodermal hepatic and ventral pancreatic domain (here referred to as HVP). In the double *fgf10*; *fgf24* mutants, *sox9b* expression is indeed lost in the HVP at 30 hpf (Fig. 4A, B). Similarly, *sox9b* expression was undetectable in the HVP of *bmp2a* morphants (Fig. 4C, D). In contrast, *sox9b* expression is not affected in the *wnt2bb* mutants (data not shown). These data demonstrate that the induction of *sox9b* in the hepatopancreatic domain at 24 hpf is controlled by FGF and BMP signals released by the lateral plate mesoderm.

Maintenance of ductal sox9b expression requires FGF and Notch signalling

A previous study has reported that *fgf10* controls HPD differentiation and maintains borders between the HPD system, the pancreas and the liver, and that the HPD system in *fgf10* mutants displays misdifferentiated cells (Dong et al., 2007). Given the similar defects in *sox9b* mutants and in the *fgf10* mutants at 3 dpf, we asked whether *fgf10* controls *sox9b* expression in the HPD system. *sox9b* early expression in the HVP is correctly induced in *fgf10* mutant at 30 hpf (data not shown). At 3 dpf, however, *sox9b* expression was barely detected in the HPD system in *fgf10* mutants (Supplementary Fig. 1A, B) though its expression is normal in the hepatic and pancreatic ductal network. To more precisely assess the role of FGF signalling on *sox9b* expression after these ductal structures are specified, wild type embryos were treated with the FGF inhibitor SU5402 from 54 to 72 hpf (Fig. 5A, B and A′, B′). In treated embryos, *sox9b* expression is strongly diminished in the EPD (Fig. 5A′, B′). As this treatment has been shown to induce endocrine differentiation from the EPD (Chung et al., 2010), the endocrine marker *neurod* was also analysed with *sox9b* expression (Fig. 5A, B). Interestingly, the ductal sites where *sox9b* expression is lost display massive endocrine differentiation. As *sox9b* mutants also harbour ectopic endocrine cells, these data strongly suggest that *sox9b* labels in the HPD system progenitors that are able to undergo endocrine differentiation. To analyse further similitude between *sox9b* and *fgf10* mutants, Nkx6.1 expression was examined in the *fgf10* mutant and compared with Pax6, as ectopic endocrine differentiation was previously reported in the HPD system of this mutant (Dong et al., 2007). Like in *sox9b* mutants, Nkx6.1 is ectopically expressed throughout the HPD system, notably within the junction with the intestine, and within the liver of *fgf10* mutants (Fig. 5C, D). Some ectopic Pax6+ endocrine cells were also detected in this region. These observations showed that (i) the HPD system of the *fgf10* and *sox9b* mutants displays a similar phenotype and (ii) *sox9b* expression is severely compromised in this structure in *fgf10* mutants, suggesting that Sox9b could mediate, at least partially, the action of FGF10 in patterning and differentiating the HPD system.

Recent studies have established that the IPD contain pancreatic Notch-responsive cells (PNCs) that correspond to pancreatic progenitors giving rise to late endocrine cells. PNCs are easily detected by expression of mCherry in the transgenic line *Tg*(*Tp1*:*hmgb1-mCherry*) (Parsons et al., 2009). Sox9 immunodetection was performed on *Tg*(*Tp1*:*hmgb1-mCherry*) larvae at 5 dpf and revealed that pancreatic ductal Sox9b is localised in the PNCs (Fig. 5G–G″). As PNCs disappear upon Notch signalling inhibition concomitant with an increase in endocrine differentiation (Parsons et al., 2009), we examined whether *sox9b* may be dependent on Notch signalling in the IPD by incubating wild type larvae with the γ-secretase inhibitor DAPT from 72 to 96 hpf. As depicted in Fig. 5E–F′, loss of *sox9b* expression in parts of the IPD of treated larvae was detected with concomitant robust increase in *neurod*+ endocrine cells at ductal sites where *sox9b* expression disappears. Collectively, our results indicate that *sox9b* expression marks the PNCs which, upon Notch inhibition, lose their *sox9b* expression and undergo endocrine differentiation. In contrast, the same DAPT treatment did not impair *sox9b* expression in the IHD (data not shown) showing that Notch is not required to maintain *sox9b* expression in the IHD.

To determine whether *sox9b* expression still labels PNCs in the adult, its expression was analysed by Q-PCR in pancreatic Notch-responsive cells isolated by FACS from *Tg*(*Tp1*:*hmgb1-eGFP*) adult fish. In the adult zebrafish pancreas, Notch activity persists in centroacinar cells (Parsons et al., 2009). In mouse, these cells display progenitor function and express *Sox9* (Rovira et al., 2010). *sox9b* expression was specifically found in Notch-responsive GFP+ cells while it was not detected at all in *ptf1a*+ acinar cells isolated from *Tg*(*ptf1a*:*eGFP*) adult fish (Fig. 5H). Conversely, the acinar marker *amylase* was not detected in GFP+ cells. This suggests that *sox9b* expression, like its murine orthologue, is localised in putative pancreatic progenitors in the adult.

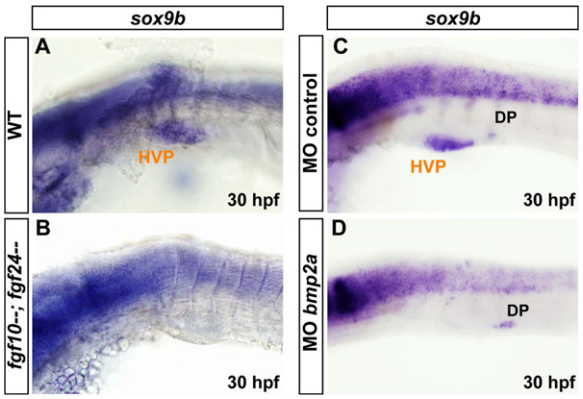

Fig. 4. *sox9b* early hepatopancreatic expression is activated by FGF and BMP and is maintained in the ducts by FGF and Notch. (A, B) *sox9b* early expression (30 hpf) in the hepatic and ventral pancreatic primordia (HVP) is not activated in compound *fgf10*; *fgf24* mutants (C, D) Similarly, *sox9b* is not induced in *bmp2a* morphants. Note that *sox9b* in the dorsal pancreatic bud (DP) is not affected.

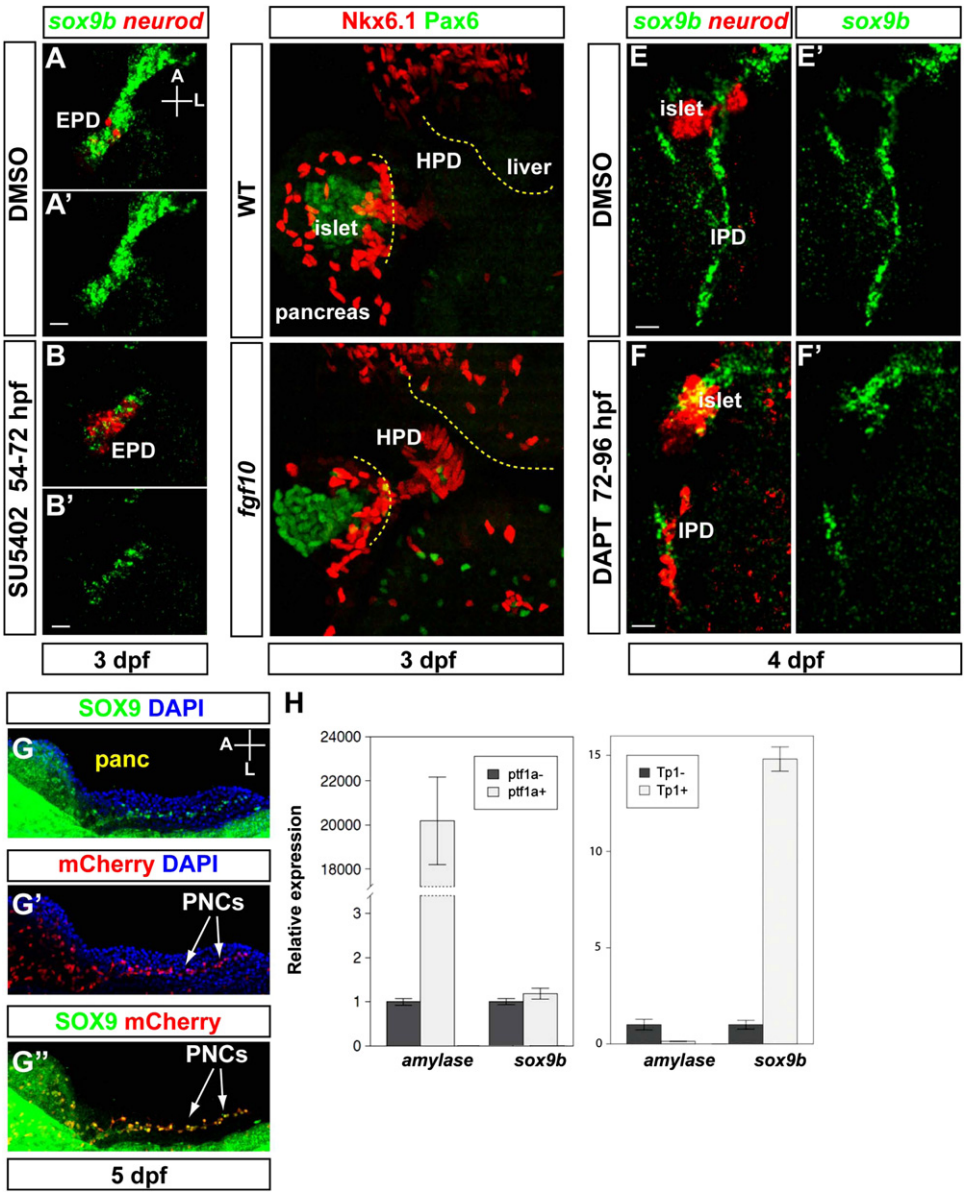

Fig. 5. Concomitant maintenance of ductal *sox9b* expression with repression of endocrine differentiation by FGF and Notch signalling. (A, B) Expression at 3 dpf of *sox9b* with the endocrine marker *neurod* in wild type embryos treated from 54 to 72 hpf with DMSO (A) or with the FGF inhibitor SU5402 (B). Confocal planes containing the EPD are shown. (A′, B′) *sox9b* expression only is shown. (C, D) Ectopic Nkx6.1+ and endocrine Pax6 cells are found in the HPD of *fgf10* mutants, as in *sox9b* mutants. Yellow dotted lines demarcate organ boundaries. (E–F′) 3-D rendering of the pancreas showing *sox9b* and *neurod* expression at 4 dpf at the level of the IPD in wild type embryos treated from 72 to 96 hpf with DMSO (E, E′) or with the Notch inhibitor DAPT (F, F′). (E′, F′) *sox9b* expression alone is shown. (G–G″) Detection of SOX9 protein in the Notch-responsive cells (PNCs) revealed with anti-mCherry in *Tg*(*Tp1*:*hmgb1-mCherry*) transgenic larvae at 5 dpf. (H) Quantitative RT-PCR analyses of *sox9b* and *amylase* expression in centroacinar and acinar cells isolated from the pancreas of adult *Tg*(*Tp1*:*hmgb1-eGFP*) (Tp1+) and *Tg*(*ptf1a*:*eGFP*) (ptf1a+) fish, respectively. EPD, extrapancreatic duct; HPD, hepatopancreatic ductal system; IPD, intrapancreatic duct. Scale bar=20 μm.

sox9b is required for late endocrine cells formation and for beta cell regeneration in larvae

In order to investigate the role of *sox9b* in differentiation of late endocrine cells, we used two experimental strategies, one stimulating precocious endocrine differentiation in the IPD by Notch inhibition as described above (Parsons et al., 2009) and the other triggering beta cell recovery upon their ablation (Pisharath et al., 2007). In the first approach, wild type and *sox9b* mutants were exposed to DAPT or DMSO from 3 to 6 dpf and endocrine differentiation in secondary islets along the IPD was examined using Pax6 and 2F11 markers at 6 dpf (Fig. 6A–D). In wild type larvae and *sox9b* mutants treated with DMSO, a few secondary endocrine cells were observed (Fig. 6A, B). In contrast, blocking Notch signalling with DAPT in wild type larvae caused significantly increased formation of secondary islets while only a few endocrine cells were detected in *sox9b* mutants (Fig. 6C, D).

In the second approach, we used the *Tg*(*ins*:*nfsB-mCherry*) line encoding in beta cells a Nitroreductase enzyme which converts Metronidazole (Met) in cytotoxins (Pisharath et al., 2007). This allows the specific and conditional beta cell ablation following incubation of larvae with Met. To compare the recovery capacity of beta cells in wild type and *sox9b* mutants, *Tg*(*ins*:*nfsB-mCherry*) larvae and *sox9b*^{fh313} mutants harbouring the (*ins*:*nfsB-mCherry*) transgene were treated with Met from 56 to 80 hpf to eliminate the dorsal bud-derived as well as the first "late" beta cells. Ablation was verified just after the treatment (Supplementary Fig. 2). While untreated wild type transgenic larvae display at

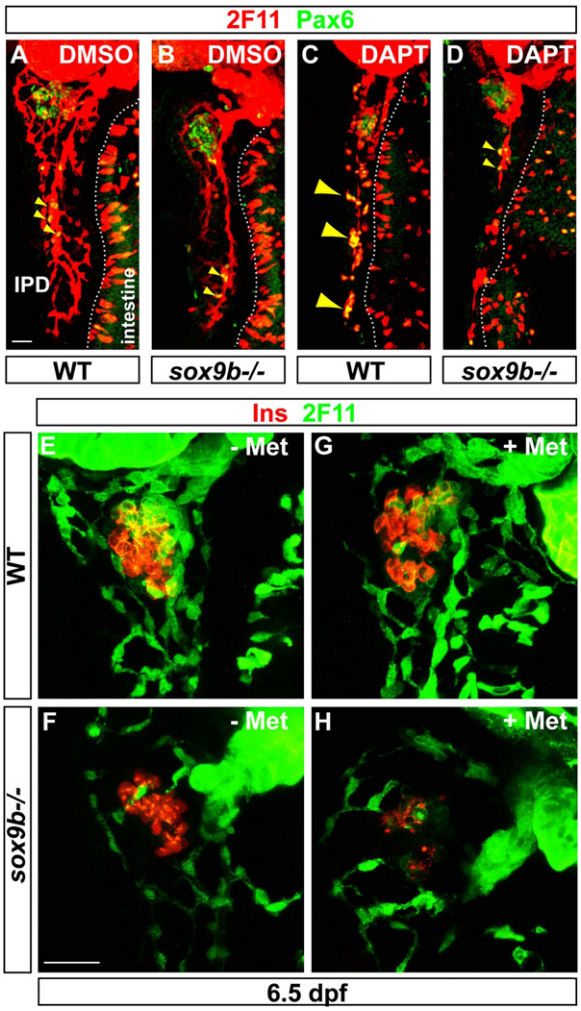

Fig. 6. *sox9b* is required for late endocrine cell formation in the IPD and for islet beta cell recovery upon ablation in larvae. (A–D) Immunodetection at 6 dpf of endocrine cell differentiation (Pax6) along the IPD labelled with 2F11 in wild type (A) and *sox9b* mutant larvae (B). Larvae were treated with DMSO (A, B) or DAPT (C, D) from 3 to 6 dpf. Secondary isolated endocrine cells are detected (small arrowheads) in wild type and *sox9b* mutant control larvae. The dotted line demarcates the intestine from the pancreatic region. (C, D) The massive increase in secondary islets resulting from DAPT treatment of wild type larvae (large arrowheads in C) is not observed in *sox9b* mutants which still display isolated endocrine cells along the IPD (small arrowheads in D). (E–H) Beta cell recovery in *Tg*(*ins*:*nfsB-mCherry*); *sox9b* larvae upon Metronidazole (Met) mediated ablation. Pancreatic ducts were labelled with 2F11 and beta cells were revealed by Insulin (Ins) immunodetection as mCherry fluorescence was undetectable after the process of immunofluorescence. (E, F) 6.5 dpf *Tg*(*ins*:*nfsB-mCherry*) wild type (E) and *sox9b* mutant larvae (F) 3 days (80 h) after DMSO treatment from 56 to 80 hpf. (G, H). *Tg*(*ins*:*nfsB-mCherry*) wild type (G) and *sox9b* mutant (H) larvae 3 days after Met exposure. Scale bar=20 μm.

least 20 beta cells clustered in the principal islet with intense mCherry fluorescence, treated larvae, as previously reported (Pisharath et al., 2007), have only very few beta cells which are also more spherical and less fluorescent, and small cell debris are often found outside the islet thereby supporting efficient ablation. The larvae were allowed to recover for 3 days after ablation in order to assess beta cell recovery (Fig. 6E–H). While the principal islet appeared completely regenerated in 32.5% of wild type larvae ($n=40$), *sox9b* mutants ($n=18$) never show normal number of beta cells. Although there is a variation of beta cell regeneration among wild type and *sox9b*−/− larvae, the number of larvae displaying absence of regeneration was dramatically increased in *sox9b* mutants (72.2%) compared to wild type (20%). Partial beta cell recovery was observed in 47.5% (5–10 beta cells/islet) wild type and in 27.8% *sox9b* mutants (1–5 beta cells/islet). This demonstrates a highly compromised recovery in *sox9b* mutants. Taken together, these two approaches demonstrate that *sox9b* is required for the formation of secondary islets along the IPD and for beta cells regeneration.

Discussion

The present work unveils an important function of zebrafish *sox9b* in hepatopancreatic duct development, in endocrine cell formation and in beta cell regeneration from pancreatic ducts. We show that *sox9b* expression appears in cells covering the interface between the hepatic and ventral pancreatic primordia and later delineates the hepatopancreatic ductal system as well as the intra-pancreatic and hepatic ducts. *sox9b* is essential for morphogenesis of the whole hepatopancreatic ducts and prevents pancreatic, hepatic and intestinal misdifferentiation in the HPD system. We also show that *sox9b* expression is induced at 24 hpf by the combined action of *fgf10* and *fgf24* and by *bmp2a*, two signalling pathways important for ventral pancreas and liver specification. In larvae, its ductal expression is maintained by FGF signalling in the HPD system and by Notch in the IPD. Finally, we show that, in the IPD, *sox9b* expression marks the PNCs in the zebrafish larva and adult, and that it is essential for the formation of secondary islets along the IPD and for beta cell regeneration in the principal islet following specific cell ablation in larvae.

sox9b marks the HPD, IPD and IHD throughout development

In wild type embryos, *sox9b* expression at 28 hpf draws an interesting pattern in the hepatopancreatic domain. Indeed, *sox9b* expression overlaps the hepatic and pancreatic primordia thereby labelling a subset of cells across each side of these two progenitor domains. As *sox9b* is detected in all hepatopancreatic ducts at later stages, this pattern suggests that the *sox9b*+ cells at 28 hpf are HPD progenitors. Recent data in mouse showed that the extrahepatobiliary system (here referred to as HPD) shares a common origin with the ventral pancreas (Spence et al., 2009). These progenitors express *Sox17* which later becomes restricted to the gall bladder. In zebrafish, *sox17* also marks the gall bladder at late developmental stages (from ~40 hpf) but its expression is not detected at 28 hpf between the hepatic and pancreatic primordia like *sox9b*. Based on our data, we propose that this *sox9b* early expression may define a progenitor cell population which gives rise to the HPD in addition to the IHD and IPD. Cell fate experiments will be required to test this hypothesis.

sox9b governs morphogenesis and differentiation of the IPD, IHD and of the HPD system

According to the restricted expression of *sox9b* in all hepatopancreatic ducts throughout development, the analysis of *sox9b*^{fh313} zebrafish larvae revealed defects specifically in these ducts and not in the pancreatic acinar tissue and the first endocrine cells nor in hepatocytes. Indeed, the pancreas of *sox9b*^{fh313} larvae display fewer, dysmorphic ducts with lower 2F11 staining. This phenotype is much less severe than the one reported for the *Sox9* pancreatic knockout mice that have a drastic pancreatic hypoplasia with reduced number of acinar, ductal and endocrine cells. In mouse, it is likely that the essential function of *Sox9* in the maintenance of the pool of pancreatic progenitors precludes the analysis of a specific requirement in duct formation at later stages. Inversely, the defects in the liver are more drastic in zebrafish *sox9b*^{fh313} mutant larvae compared to *Sox9* hepatic knockout mice. Indeed connections between duct biliary cells

leading the formation of the IHD network are severely disrupted at 7 dpf, when the biliary system is completed and functional in wild type larvae, while hepatic duct maturation is only delayed in the mouse *Sox9* knockout and bile ducts are normal 5 weeks after birth (Antoniou et al., 2009). This emphasises the benefit to examine two animal models to gain complete understanding of the function of a regulatory gene. The phenotypic differences between the *Sox9* loss-of-function in mouse and zebrafish are probably due to the involvement of other factors able to compensate one another in the pancreas or liver.

In the zebrafish liver, IHD development does not proceed via a ductal intermediate as described in mouse (Lorent et al., 2004). Crucial steps of zebrafish IHD morphogenesis, occur between 2.5 and 4 dpf, and involve formation of a network by the first contiguous IHD cells which then undergoes extensive morphogenetic remodelling: IHD cells adopt a stellate morphology, migrate and separate from one another starting to arrange in a network, the connections between them narrow, they proliferate extensively and lumen forms. Our data show that *sox9b* regulates formation of intercellular connections but not proliferation. It would be interesting to explore whether duct cell migration and lumen formation are also affected in the mutants.

Despite differences in the IHD morphogenesis and anatomy between species, there is strong conservation of the molecular processes leading IHD development, including the Notch pathway. Particularly, it has been shown in zebrafish that Notch signalling is required for IHD remodelling (Lorent et al., 2010). In mouse, a link between Sox9 and Notch in biliary development has been suggested based on the observation of the expression pattern of *Hes1*, a mediator of Notch signalling, is affected in *Sox9* hepatic knockout (Antoniou et al., 2009). In zebrafish, it is not clear whether *sox9b* and Notch signalling cooperate in IHD morphogenesis as (i) Notch appears to regulate more aspects of duct remodelling than *sox9b* and (ii) *sox9b* is still well expressed in the IHD following DAPT treatment (data not shown). Overall, our data suggest a role of *Sox9* in IHD maturation being conserved through the vertebrates and the relation between *Sox9* and Notch signalling in zebrafish as well as in mouse remains to be elucidated.

In the zebrafish pancreas, duct morphogenesis is not well understood though it has been proposed that pancreatic ducts arise from ductal progenitor cells *in situ* rather than arising from reiterative branching of the pancreatic epithelium, like in mammalian pancreas (Yee et al., 2005). As our results reveal that *sox9b* is involved in pancreatic duct cell differentiation and morphogenesis, it would be interesting to determine whether Sox9 function in pancreatic duct development is also conserved in mammals. Taken together, our results uncovering the importance of *sox9b* in both pancreatic and hepatic duct morphogenesis suggest common mechanisms involved in duct development in both organs.

We also demonstrate that *sox9b* is essential for the proper morphogenesis and differentiation of the HPD system connecting the liver and pancreatic ducts to the intestine. Indeed, in *sox9b* mutants, the HPD structures are dysmorphic and misdifferentiated as highlighted by (i) enlarged and less well morphologically defined HPD structures, (ii) smaller gall bladder, and (iii) presence of ectopic hepatocyte and pancreatic markers and lower ductal marker 2F11 staining. Ectopic pancreatic acinar differentiation was never detected in the HPD of *sox9b* mutants suggesting that these ducts do not have the competence to differentiate into all cell types or that signalling pathways from adjacent tissues have a repressive role on acinar differentiation from these progenitors. Alternatively, one could argue that ectopic endocrine cells and hepatocytes along the HPD have migrated from the pancreas and the liver. However, the presence of chimeric differentiated cells (expressing endocrine pancreatic and hepatic markers together in the same cells) within the HPD system in *sox9b* mutants argues for genuine misdifferentiation of HPD cells. Misdifferentiation in the HPD system at 3 dpf in the absence of *sox9b* indicates that *sox9b* is required to maintain ductal identity and the boundaries between organs in the HPD. In mouse, ectopic pancreas in the HPD system as well as GB agenesis have been described in *Hes1* knockout embryos (Sumazaki et al., 2004). However, while only ectopic endocrine cells were detected in the HPD of *sox9b* mutants, both ectopic endocrine and exocrine tissues were found in the HPD of *Hes1* mice. In contrast, the defects of the HPD system presented by *sox9b* mutants are mostly similar to the zebrafish *fgf10* mutant, strongly suggesting that *sox9b* and *fgf10* are acting in the same regulatory pathway. This is further supported by the loss of *sox9b* expression in the HPD system of *fgf10* mutants.

The ductal defects described here for the *sox9b*^{fh313} nonsense mutation were also observed in the previously described *sox9b*^{b971} mutant (data not shown) (Yan et al., 2005). However, this *sox9b*^{b971} mutant is not the adequate tool to address the specific function of *sox9b* as (i) this allele contains a large deletion removing *sox9b* but also *sox8* and *pvalb1, 4, 8* (Yokoi et al., 2009) and (ii) the global morphology of *sox9b*^{b971} mutants was altered and many organs were hypoplasic such as the pancreas and liver, but also the intestine and swim bladder which both do not express *sox9b*. Moreover, we can assume that *sox9b*^{fh313} is a null mutant as the compound *sox9b*^{fh313}; *sox9b*^{b971} mutants (obtained by crossing *sox9b*^{fh313} and *sox9b*^{b971} heterozygous) present the same defects as *sox9b*^{fh313} homozygous (data not shown), and SOX9 protein could not be detected in the *sox9b*^{fh313} homozygous.

sox9b is required for "late" endocrine cells formation and regeneration in the larva

In zebrafish larvae, the first "late" endocrine cells derive from the EPD at around 3 dpf (Field et al., 2003). The failure of *sox9b* mutants to restore the beta cell mass in the principal islet after ablation indicates that *sox9b* is required for the capacity of ductal cells to generate the secondary transition of endocrine cells. It is plausible that *sox9b* is crucial to preserve the progenitor pluripotency of ductal cells. Previous studies have reported *Sox9* expression in adult human and mouse HPD system (Furuyama et al., 2011) and demonstrated that the HPD cells of adult human donors express *SOX9* and can generate hepatic and pancreatic cell types *in vitro* (Cardinale et al., 2011). Hence we can speculate that *sox9b* inactivation leads to a premature cell differentiation causing a depletion of progenitor cells in the EPD. This hypothesis is supported by the presence of misdifferentiated cells in the HPD of *sox9b* mutants.

A role of *sox9b* in maintenance of the progenitor state is further strengthened by our observation that FGF and Notch signalling, both required to maintain progenitor identity (Dong et al., 2007; Wang et al., 2011), also maintain *sox9b* expression in the EPD and IPD, respectively. Furthermore, we show that *sox9b* marks the PNCs in the larva and that it mediates Notch repressive activity on endocrine differentiation in the IPD. These PNCs constitute progenitors that have recently been identified along the IPD and have been shown to be able to contribute to adult endocrine cells in zebrafish (Parsons et al., 2009; Wang et al., 2011). Our results showing that *sox9b* is required for IPD development suggest that *sox9b* is important to establish pancreatic progenitors in the IPD. In the adult, PNCs correspond to centroacinar cells, a cell type closely associated to terminal ducts that has been shown in mouse to be capable of endocrine differentiation *in vitro* (Rovira et al., 2010). Thus, our observation that *sox9b* expression localises in PNCs in adult zebrafish suggests that *sox9b* could play progenitor function in beta cell genesis in adult

pancreas. Future experiments will be needed to explore this possibility.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.04.002.

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